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ORIGINAL PAPER

Appendix B

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Doxycycline-induced transgene expression during Drosophila development and aging

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Abstract The "reverse" tetracycline repressor (rtR) binds a specific DNA element, the tetracycline operator (tetO), only in the presence of tetracycline, or derivatives such as doxycycline (dox). Fusion of rtR to the transcriptional activation domain of herpes virus protein VP16 produces a eukaryotic transactivator protein (rtTA). rtTA has previously been shown to allow dox-dependent transcription of transgenes linked to tetO sequences in mammals. To adapt this system to *Drosophila*, the *Actin5C* promoter was used to drive constitutive expression of rtTA in transgenic flies. Three reporter constructs, each encoding *E. coli* β-galactosidase (β-gal), were also introduced into transgenic flies. In one reporter seven tetO sequences were fused to the *Adh* core promoter. The other two reporter constructs contain seven tetO sequences fused to the *hsp70* core promoter. Feeding of transgenic *Drosophila* containing the rtTA construct and any one of the three reporter constructs with dox caused up to 100-fold induction of β-gal. Dox induced β-gal expression in all tissues, in larvae and in young and senescent adults. Induction of β-gal in adults had no detectable effect on life span. These results suggest the potential usefulness of this system for testing specific genes for effects on *Drosophila* development and aging.

Key words Tetracycline repressor · Inducible promoter · Drosophila · Aging

Introduction

Inducible gene expression systems have long been an important tool in analyzing the function of specific genes in bacteria, yeasts, and *Drosophila*. In *Drosophila*, inducible transgenic systems usually rely wholly or in part on the use of a heat shock protein (hsp) gene promoter, which is transcriptionally induced in response to heat stress (Lis et al. 1983). While hsp gene promoters have been used to great advantage in many experiments, the system has several important limitations. First, the heat stress required for induction can have pleiotropic effects, including developmental abnormalities (phenocopies) (Lindquist 1986) and reduced fertility and viability. This is a problem particularly in experiments designed to study the aging process, since life span will be dramatically affected by changes in fertility and viability (Tower 1996). Another situation where the use of a heat-inducible promoter is problematic is the analysis of the heat shock proteins themselves. It is not possible to induce expression of a single hsp and study its effects, without the complication of inducing the entire endogenous repertoire of hsps. This problem has been overcome in certain experiments by using the metallothionein promoter (Petersen and Lindquist 1988; Solomon et al. 1991), which is inducible by heavy metal ions. However, the metallothionein promoter functions only in specific gut cells in transgenic *Drosophila* (Otto et al. 1987), thus limiting its potential usefulness. Therefore, there is a need for an alternative inducible gene expression system in *Drosophila*.

In the last five years, efficient inducible gene expression systems have been developed for mammalian systems based on the *E. coli* tetracycline repressor (tetR) (Gossen and Bujard 1992; Furth et al. 1994). The tetR binds to its target sequence, the tetracycline operator (tetO) only in the absence of the antibiotic tetracycline. The first system developed for mammals was the "tet-off" system (Gossen and Bujard 1992; Furth et al. 1994; Shockett et al. 1995). tetR protein was fused with the transcriptional activation domain of herpes virus transcription factor VP16. In the absence of tetracycline this protein binds to tetO sequences placed within the promoter of a gene of interest, thereby driving transcription. Addition of tetracycline then prevents binding and stops transcription ("tet-off"). A "tet-on" system was created

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by generating a mutant tetR:VP16 fusion protein, which had the reverse property of only binding to the tetO and activating transcription in the presence of tetracycline ("tet-on") (Gossen et al. 1995; Kistner et al. 1996). We report here the successful adaptation of the "tet-on" system to transgenic *Drosophila*.

Materials and methods

Plasmid constructions

Plasmid rtTA (reverse-tetracycline Trans Activator) was constructed by first inserting the 850-bp *Hin*dIII-*Xba*I fragment from pCaSpeR-AUG/β-gal (Thummel et al. 1988), containing the SV40 splice and poly(A) signals, into the *Hin*dIII (partial restriction digestion) and *Xba*I sites of the polylinker of the pCaSpeR4 transformation vector (Thummel and Pirotta 1992), to generate plasmid cSV. Plasmid pUHD172–1neo (Gossen et al. 1995) was digested with *Eco*RI, endfilled with T4 polymerase, then digested with *Bam*HI, to liberate a 1-kb fragment containing the reverse-tetracycline trans-activator coding sequence. Plasmid cSV was digested with *Spe*I, endfilled with T4 polymerase, then digested with *Bam*HI, and the 1-kb fragment from pUHD172–1neo was inserted, to generate the plasmid cTSV. DNA sequencing of cTSV revealed that it had resulted from an unexpected ligation event: the *Eco*RI site from the inserted fragment was conserved in this cloning step, and the 1-kb fragment was actually inserted into the *Bam*HI site, without any change in the *Spe*I site. The *Actin5C* promoter was inserted into plasmid cTSV in several steps. First, plasmid D237 (also called "Act5C > Draf+ > nuc-lacZ"; Struhl and Basler 1993) was digested with *Not*I, endfilled with T4 polymerase, then digested with *Kpn*I, and the resultant 4.3-kb fragment containing the *Actin5C* promoter was inserted into the *Kpn*I/*Eco*RV sites of pBlueScript II KS (Stratagene), to generate pAc. The 4.3-kb *Actin5C* promoter fragment was liberated from pAc by restriction digestion with *Kpn*I and *Eco*RI, and inserted into the *Kpn*I/*Eco*RI sites of cTSV, to generate the plasmid cATSV. DNA sequencing revealed that the *Actin5C* promoter in plasmid cATSV was in the wrong orientation relative to the reverse-tetracycline transactivator coding region. To correct this, the *Actin5C* promoter region was liberated by digestion with *Eco*RI, and then re-inserted into the same *Eco*RI site. DNA sequencing was used to identify a construct with the *Actin5C* promoter in the correct orientation, which was then named plasmid rtTA.

The seven tandem repeats of the tetO region in plasmid pUHC13–3 (Gossen et al. 1995) were amplified by PCR using the primers: 5′-TCGACTGCAGCTTTCGTCTTCAAGAATTCCTCGAG-3′ and 5′-AGCTTCTAGATACACGCCTACTCGACCCGGGTACCGAG-3′. The 367-bp PCR product was digested with *Pst*I and *Xba*I at the sites engineered into the primers, and then inserted into the *Pst*I/*Xba*I sites of pBlueScript II, to generate plasmid p7T.

Plasmid 7TAdh was constructed as follows. Plasmid pAdh/β-gal (Irvine et al. 1991; Koelle et al. 1991) was partially digested with *Eco*RI, and then completely digested with *Pst*I to liberate a 4.8-kb fragment containing the *Adh* basal promoter region (positions −33 to +53), the *Ubx* 5′ leader sequences fused to *lacZ*, and the SV40 splice and poly(A) signals. This fragment was cloned into the *Pst*I/*Eco*RI sites of the pCaSpeR4 polylinker, to generate plasmid pCaSpeR-Adh/β-gal. The 359-bp *Pst*I-*Xba*I fragment from plasmid p7T, containing the heptameric tetO region, was then inserted into the *Pst*I/*Xba*I sites of pCaSpeR-Adh/β-gal, to generate plasmid 7TAdh.

Plasmid 7T40 was constructed as follows. Construct c70Z (Simon and Lis 1987) was digested with *Hin*dIII and *Eco*RI to liberate a fragment containing the *hsp70* promoter fused to *E. coli lacZ*. This *Hin*dIII-*Eco*RI fragment was cloned into the *Hin*dIII/*Eco*RI sites of plasmid pBS2N to generate plasmid pBS2N′. Plasmid pBS2N is pBlueScript II KS + (Stratagene) in which the unique *Kpn*I site has been converted to a *Not*I site (a gift of L.R. Bell, University of Southern California). Construct c70Z was also digested with *Eco*RI alone to liberate an *Eco*RI fragment containing the *hsp70* poly(A) signal sequences, and this fragment was cloned into the unique *Eco*RI site of plasmid pBS2N′ to generate plasmid pBS2N″. Plasmid pBS2N″ was digested with *Hin*dIII and *Apa*I, treated with exonuclease III and with nuclease S1, and then ligated. The resultant plasmid was called c40Z, and DNA sequencing revealed a 5′ *hsp70* promoter deletion to position −40 relative to the start site of transcription. Plasmid c40Z is one of a series of *hsp70* 5′ promoter deletions which will be described in detail elsewhere (J. C. Wheeler and J. Tower, unpublished data). Plasmid c40Z was digested with *Not*I to liberate a 3.7-kb fragment containing the entire 5′Δ-40 *hsp70:lacZ* fusion gene, and this fragment was cloned into the *Not*I site of p7T, to generate plasmid p7T40-pre. A fragment containing the seven tetO repeats and the entire 5′Δ-40 *hsp70:lacZ* fusion gene was liberated from p7T40-pre by digestion with *Xho*I and *Spe*I, then inserted into the *Xho*I/*Spe*I sites in the polylinker of pCaSpeR4, to generate plasmid 7T40.

Plasmid 7TAUG was constructed as follows. A 4.6-kb SalI fragment from pCaSpeR-AUG/β-gal (Thummel et al. 1988), containing the *Adh* translation initiation sequence fused to *lacZ* and the SV40 splice and poly(A) signals, was cloned into the *Sal*I site of pBlueScript II KS, to generate plasmid pAUG. A *Pst*I fragment from plasmid 7T40, containing the seven tetO repeats and the *hsp70* promoter from −40 to +86, was inserted into the *Pst*I site of pAUG, to generate plasmid p7TAUG. A fragment containing the seven tetO repeats and the entire *hsp70:lacZ* fusion gene was liberated from p7TAUG by digestion with *Xho*I, and inserted into the *Xho*I site of pCaSpeR4, to generate plasmid 7TAUG.

Drosophila culture

Fly stocks were maintained on cornmeal/agar medium (Ashburner 1989). To obtain adult flies of defined ages, stocks were cultured at 25° C until 0–2 days post-eclosion, and then males only were transferred to 25° C or 29° C as indicated in Figure legends. These males were maintained at < 50 per vial and transferred to fresh vials every 2–4 days. Double transgenic adult males were obtained by crossing males of a transactivator stock (rtTA) to virgins of the reporter stocks (7TAdh, 7T40, and 7TAUG). Transgenic flies were generated by standard methods (Rubin and Spradling 1982), using the w^{1118} recipient strain.

Doxycycline treatments

Young flies (5–7 days post-eclosion) and old flies (28–32 days post-eclosion) were treated with the tetracycline derivative doxycycline hydrochloride (dox) (Sigma) by feeding. The indicated concentration of dox, in 20 mM Tris (pH 7.5) containing 10% sucrose, was soaked into a single Kim-Wipe (Kimberly-Clark), in an empty *Drosophila* culture vial. After feeding with dox for the specified time, the flies were returned to cornmeal/agar food vials, and allowed to recover as indicated. For treatment of larvae, the cornmeal/agar medium was supplemented with dox to a final concentration of 0.25 mg/ml, prior to seeding of the culture.

Spectrophotometric assay of β-galactosidase activity

β-Galactosidase (β-gal) activity was quantitated in whole fly extracts using published procedures (Simon and Lis 1987). Assays were performed under conditions in which the reaction was linear with regard to the amount of extract. Data are presented as the average +/− the standard deviation for triplicate assays. Protein concentration of extracts was determined using the Bradford reagent (BioRad). The w^{1118} strain was used to generate all transgenic lines, and no β-gal activity was detectable in extracts of the w^{1118} strain using the spectrophotometric assay.

In situ staining for β-galactosidase activity

β-galactosidase expression was visualized in dissected flies, larvae, and cryostat sections using published procedures (Simon et al. 1985).

Results

Basic components of the system

To achieve tetracycline-inducible induction of transgenes in all tissues it is necessary that the reverse tetracycline transactivator (rtTA) be expressed in all tissues. The rtTA is a fusion of the reverse tetracycline receptor (rtR), which binds to DNA only in the presence of tetracycline, with the transcriptional activation domain of herpes virus transcription factor VP16. In construct rtTA, the constitutive *Drosophila Actin5C* promoter was used to drive expression of the rtTA coding region. This construct also contains the SV40 poly(A) signal sequence (Fig. 1A). To test the system, three reporter constructs were generated, each encoding *E. coli* β-gal. The constructs differed in the source of the core promoter, 5′ UTR, and polyadenylation signal sequences in order to maximize the chances of generating a construct which could yield high-level transgenic protein expression in *Drosophila*. In the first reporter construct (7TAdh) seven tetO sequences are fused to the *Adh* core promoter, followed by the *Ubx* 5′ untranslated region, the *E. coli lacZ* coding region, and the SV40 poly(A) signal (Fig. 1B). A regulatory element composed of seven tetO sequences was chosen because this element was previously shown to function in transgenic mice (Kistner et al. 1996). In the second reporter (7T40), the seven tetO sequences are fused to the *hsp70* core promoter, and *hsp70* 5′ untranslated region, followed by the *E. coli lacZ* coding sequences and the *hsp70* poly(A) signal (Fig. 1C). In the third construct (7TAUG), the seven tetO sequences are fused to the *hsp70* core promoter, followed by the *Adh* 5′ untranslated region, the *lacZ* coding region, and the SV40 poly(A) signal (Fig. 1D). Multiple independent transgenic lines were generated for each construct. Each line is homozygous for the transgenic construct, and is designated by the name of the construct followed by the chromosome in which the construct is inserted (in parenthesis), followed by a letter/number combination for each independent transgenic line. For example, line 7TAdh(2)A2 is transgenic line number A2 and has the 7TAdh construct inserted on the second chromosome.

Flies were then generated which contained both the rtTA construct and the 7TAdh construct ("double-transgenic" flies). This was done by crossing flies of stock rtTA(2)C1 to flies of stock 7TAdh(2)A2, which yields progeny containing one copy of each construct. A sample of these double transgenic flies were fed sucrose solution containing 1.0 mg/ml dox for 48 h, while the controls were fed sucrose solution alone. The flies were allowed to recover for 3 days, then sectioned using a cryostat, and the sections were stained for β-gal activity (Fig. 2A). In the treated flies robust β-gal activity (blue stain) was detected in all tissues. In the control flies, low-level β-gal activity was detected primarily in the gut, and thus the system allows dox-induced transgene expression in all tissues of the adult. The same results were obtained with transgenic flies containing the other two reporter constructs, 7T40 and 7TAUG (data not shown).

To determine if the system also works during development, line rtTA(2)C1 was crossed again to reporter line 7TAdh(2)A2, and also to reporter line 7T40(3)B1, and the larvae from each cross were cultured on food containing 0.25 mg/ml dox, and on

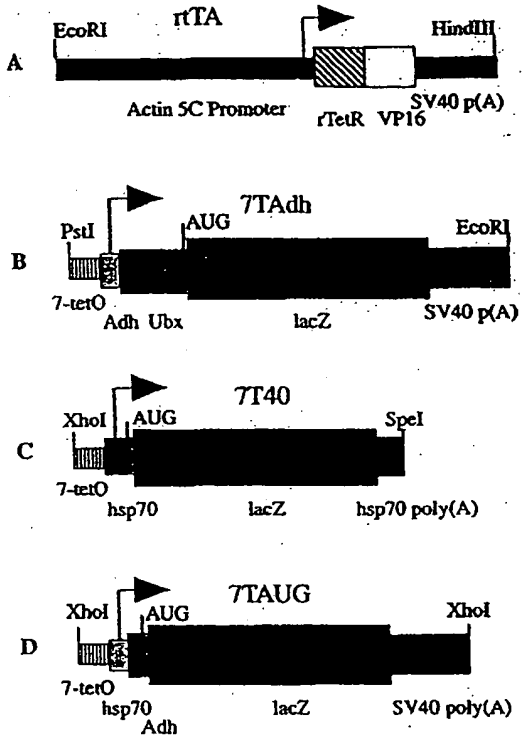

Fig. 1A–D Transgenic constructs. Each construct fragment shown is cloned into the indicated restriction sites of the polylinker of the pCaSpeR-4 transformation vector. The assembly of each construct is described in detail in Materials and methods. Diagrams are not to scale. A rtTA. The constitutive *Actin5C* promoter and 5′ untranslated region are fused to the coding sequences for the rtTA (reverse tetracycline transactivator), which is a fusion of the rtR (reverse tetracycline repressor) and the transcriptional activation domain of herpes virus protein VP16. The poly(A) signal sequences are from SV40. B 7TAdh. Reporter construct consisting of seven tetO sequences, the *Adh* core promoter, the *Ubx* 5′ untranslated region and translational initiation sequence, the *E. coli lacZ* coding region and the SV40 poly(A) signal sequences. C 7T40. Reporter construct consisting of seven tetO sequences, the *hsp70* core promoter, 5′ untranslated region and translational initiation sequence, the *E. coli lacZ* coding region and the *hsp70* poly(A) signal sequence. D 7TAUG. Reporter construct consisting of seven tetO sequences, the *hsp70* core promoter, the *Adh* 5′ untranslated region and translational initiation sequence, the *E. coli lacZ* coding region, and the SV40 poly(A) signal sequence

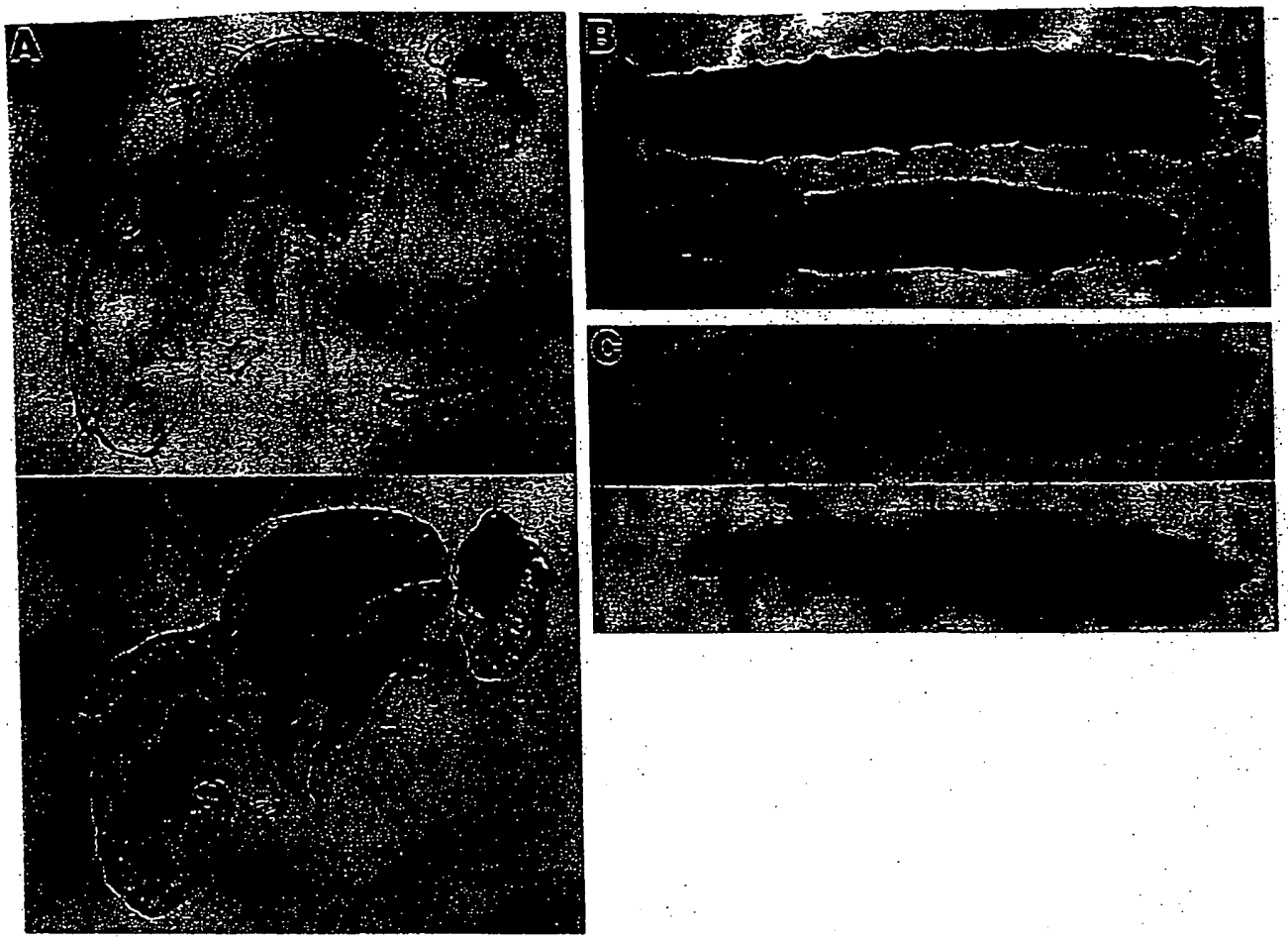

Fig. 2A–C Dox-induced transgene expression detected by an in situ β-gal activity assay. A rtTA transgenic line rtTA(2)C1 was crossed to reporter line 7TAdh(2)A2. Young adult progeny were fed with either control sucrose solution (*upper panel*) or sucrose solution containing 1.0 mg/ml dox (*lower panel*) for 48 h, and then allowed to recover for 3 days. Flies were sectioned on a cryostat, and stained for β-gal activity using the chromogenic substrate X-gal. In the control (*upper panel*), low-level β-gal activity is detected primarily in gut tissues. The gut staining indicates some leakiness of expression in the absence of dox, as in non-transgenic *Drosophila* only very faint gut staining is detectable, and only in the abdomen (data not shown, see also Wheeler et al. 1995). In dox-treated (*lower panel*), β-gal activity is detected in all tissues, with the exception of the central region of the indirect flight muscles. All of the indirect flight muscle tissue stains intensely if the staining reaction is allowed to continue for a longer period (data not shown). However with longer staining times the increased intensity of stain in the other body segments obscures the detail of specific tissues, and therefore the results for the shorter staining time are presented. B Progeny from the cross rtTA(2)C1 × 7T40(3)B1 were cultured on standard *Drosophila* culture media (upper larva) or *Drosophila* media containing 0.25 mg/ml dox (lower larva). Whole third-instar larvae were stained in situ for β-gal activity. No β-gal activity was detected in the control tissue larvae (*upper larvae*), or in non-transgenic larvae (data not shown). General β-gal activity was detected in the dox treated larvae (*lower larvae*). C Repeat of the experiment in B, using progeny of the cross rtTA(2)C1 × 7TAdh(2)A2. β-Gal expression in larvae with this reporter was reproducibly less efficient than in the experiment shown in B

control food. As seen in Fig. 2B, C, staining of whole third-instar larvae revealed high-level, tissue general induction of β-gal activity with reporter 7T40(3)B1, and somewhat lower level, tissue general induction with reporter 7TAdh(2)A2. The dox-fed larvae were also observed to be slightly smaller than the controls, which may be due to a toxic effect of the dox and/or β-gal expression during development.

Characterization of the response

The induction of β-gal expression can be quantitated by spectrophotometric assay of β-gal activity in fly extracts. This assay was used to optimize the time course of dox treatment. Transactivator line rtTA(2)C1 was crossed to reporter line 7TAdh(3)D1, and the double transgenic progeny were treated with 1.0 mg/ml of dox for 24 h, 48 h, and 48 h plus varying times of recovery without dox. As seen in Fig. 3A, 48 h of treatment plus 3 days of recovery gave the optimal degree of induction (~10-fold). With greater times of recovery, β-gal activity decreased, indicating that the induction is reversible upon withdrawal of dox. The same result was obtained using a different reporter stock, containing the 7T40

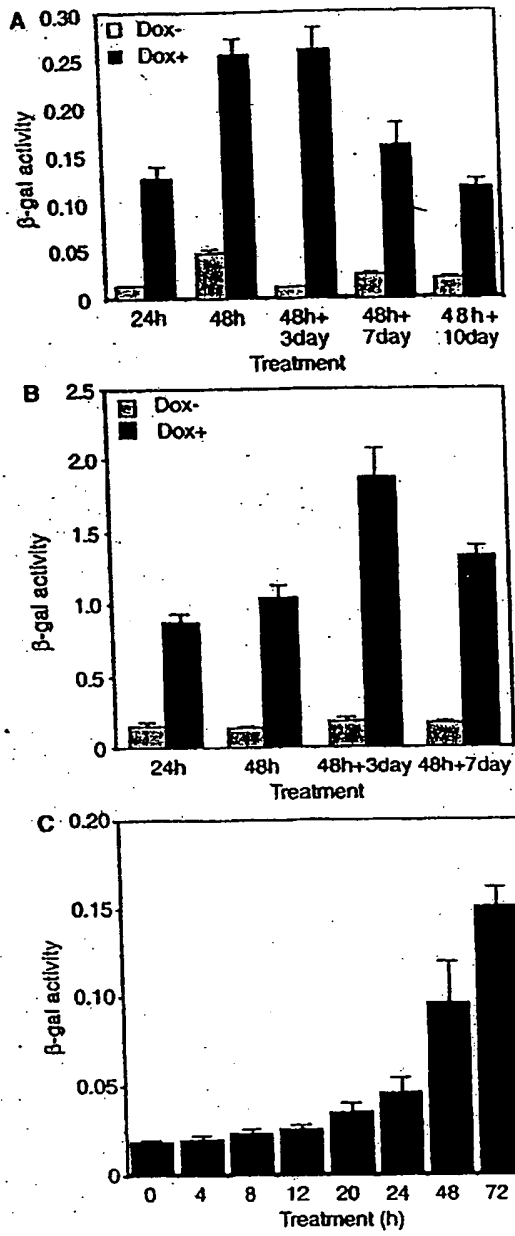

Fig. 3A–C Time course of transgene induction by dox. A Young adult progeny of the cross rtTA(2)C1 × 7TAdh(3)D1 were mock treated (Dox-; *stippled bars*), or treated with 1.0 mg/ml dox (Dox+; *black bars*) for the indicated time periods, and allowed to recover as indicated. Triplicate samples containing three flies each were homogenized, and β-gal activity was quantitated using the spectrophotometric assay. β-Gal activity is expressed in relative units, and the averages +/- SD are presented. B The experiment in A was repeated with a different reporter stock, using young adult progeny from cross rtTA(2)C1 × 7T40(2)E1. C The experiment in A was repeated using progeny from cross rtTA(2)C1 × 7TAdh(2)A2, and the timecourse for induction was analyzed in greater detail

construct, 7T40(2)E1 (Fig. 3B). Thus, both the *hsp70* core promoter and the *Adh* core promoter can respond to activation by the tetO sequences and the rtTA transactivator.

In transgenic mice the activation by the rtTA transactivator can be quite rapid, with activation by several orders of magnitude occurring in the first 4 h, and maximum levels of activation being achieved by 24 h (Kistner et al. 1996). The timecourse of activation in *Drosophila* was analyzed in greater detail (Fig. 3 C), and found to be significantly slower. In the progeny of the cross rtTA(2)C1 × 7TAdh(2)A2, induction of β-gal by dox feeding was quantitated at intervals between 4 and 72 h. Significant activation was not detected until 8–20 h, and maximal induction required ≥72 h. Similar results were obtained with construct 7TAUG (data not shown). Note that while the level of induction and timecourse was similar for the different reporters in Fig. 3, they are not identical. This probably reflects small differences in the activities of the different reporter insertions, as well as the variability inherent in working with live adult *Drosophila* and administration of dox by feeding.

The *Drosophila* tet-on system was next characterized for the dose response to dox (Fig. 4). Double transgenic adults (rtTA(2)C1 × 7TAdh(2)A2) were fed dox for 48 h and allowed to recover for 3 days (Fig. 4, open circles), or for 96 h plus a 3-day recovery period (closed circles). For 48-h treatment times, β-gal activity was found to increase in response to dox concentrations from 0.01 to 2 mg/ml. Use of the longer 96-h treatment time allowed equivalent levels of β-gal expression with one-tenth as much dox. Thus, longer treatment times reduce the amount of dox required for efficient induction.

To compare the relative activities of the three different reporter constructs, two independent transgenic lines for each reporter were crossed to the rtTA(2)C1 transactivator line (Fig. 5A). Dox-induced β-gal expression

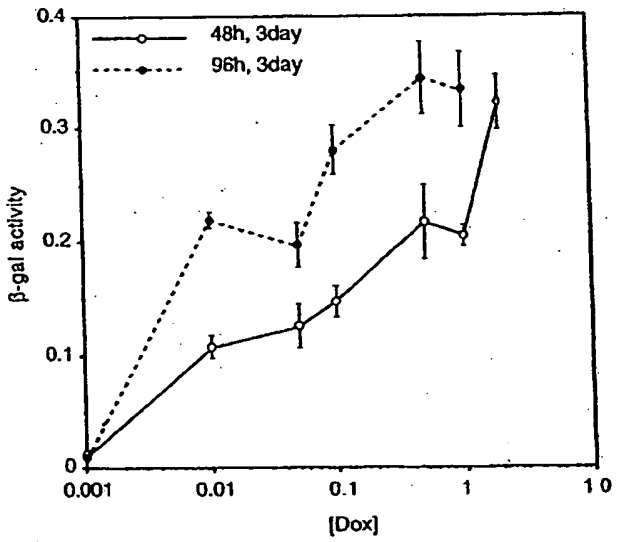

Fig. 4 Dose response of transgene induction by dox. Young adult progeny of cross rtTA(2)C1 × 7TAdh(2)A2 were fed the indicated concentrations of dox for 48 h and allowed to recover for 3 days (*open circles*), or for 96 h plus a 3-day recovery period (*closed circles*). β-Gal expression was quantitated as in Fig. 3

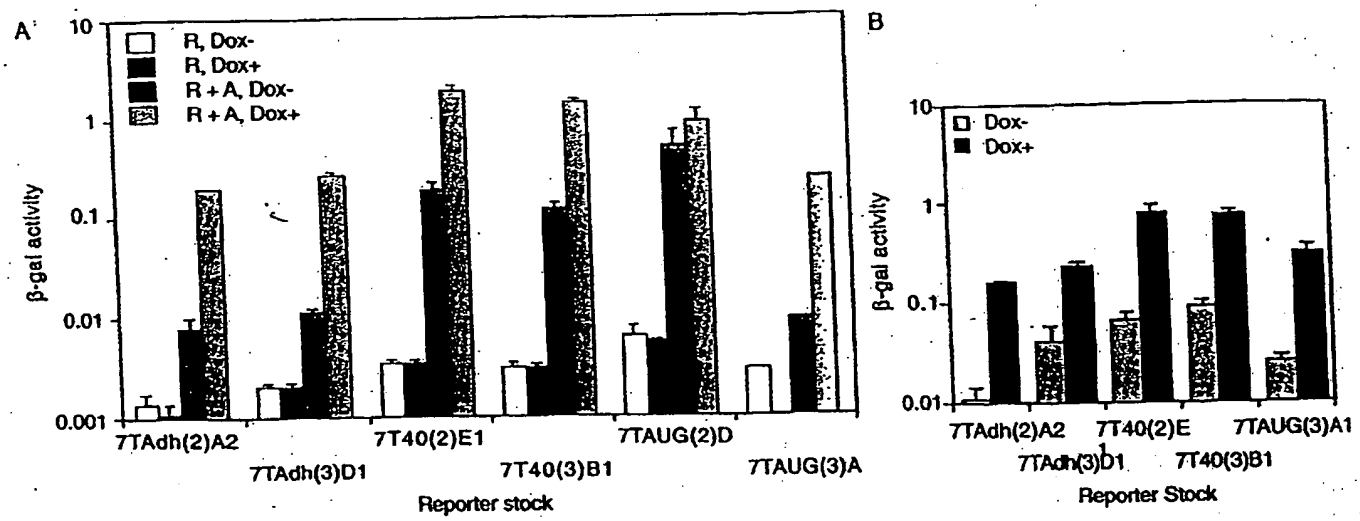

Fig. 5A, B Comparison of transgenic reporter constructs and lines. All dox treatments were for 48 h plus 3 days recovery. A Assay in young adults. The indicated reporter lines (R, reporter alone) were assayed with and without dox treatment, as indicated. Each indicated reporter line was also crossed to the rtTA line rtTA(2)C1, and the progeny (R+A, reporter plus activator) were assayed with and without dox treatment, as indicated. B Assay in old adults. rtTA line rtTA(2)C1 was crossed to each indicated reporter line, and old adult progeny were assayed with and without dox treatment, as indicated. β-Gal expression was quantitated as in Fig. 3

was observed with all three constructs, with induction factors ranging from 12- to 25- fold. In general, the 7T40 reporter construct gave higher levels of β-gal expression than the other two reporter constructs; however, the background expression in the absence of dox was also higher. Thus, the induction factor achieved was similar for each of the three reporter constructs.

To determine if the system functions during aging of *Drosophila*, the activity of each reporter construct was also assayed in senescent (30-day-old) flies (Fig 5B). Each reporter was found to support dox-induced β-gal expression in senescent flies, with induction factors ranging from 8- to 15-fold.

The dox-inducible system is dependent upon efficient, general expression of the transactivator construct, rtTA. Because the chromosomal site of insertion of the rtTA transgene can affect the level of expression, different independent rtTA transgenic lines may vary in their activity. To compare their activities, each of 13 independent rtTA transgenic lines was crossed to the 7TAdh(3)D1 reporter, and the efficiency of induction of β-gal by dox was quantitated (Fig. 6A). The different independent rtTA lines were found to vary in activity, both with regard to the amount of background β-gal activity in the absence of dox, and with regard to the maximum level of induction in the presence of dox. Transgenic transactivator line rtTA(3)E2 appeared to be the best: in the absence of dox, background β-gal levels were as low as in flies carrying the reporter construct in the absence of any transactivator, and dox treatment yielded a 40-fold induction. To confirm this result, each

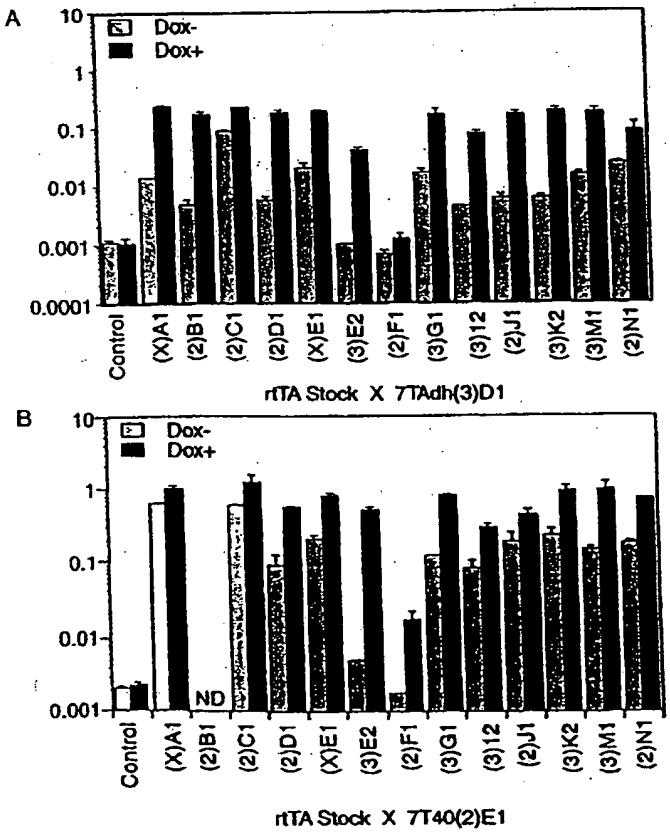

Fig. 6A, B Comparison of different transgenic transactivator (rtTA) lines. All dox treatments were for 48 h plus 3 days recovery. A The indicated rtTA lines were each crossed to reporter line 7TAdh(3)D1. The young adult progeny from each cross were assayed without dox treatment (Dox-; *stippled bars*), and with 1.0 mg/ml dox treatment (Dox+; *black bars*), as indicated. Control was the reporter line 7TAdh(3)D1 alone. B The experiment in A was repeated using the reporter line 7T40(2)E1. ND, not done

transactivator stock was also tested in combination with reporter stock 7T40(2)E1 (Fig. 6B). Again the various transactivator lines varied with regard to background and maximal level of induction, and their activity relative to each other was similar to that observed using the 7TAdh reporter. Line rtTA(3)E2 was again found to have the lowest background, and to be the most active, yielding 100-fold induction of β-gal in response to dox.

One potential use of the dox-inducible system in *Drosophila* is in the analysis of the effects of specific genes on the aging process. Ideally, for such experiments, the system itself should not have any effect on life span. To characterize the system for effects on life span, several genetic backgrounds were tested for longevity, with and without dox feeding. Wild-type flies exhibited no negative effects on life span when fed with 0.1 mg/ml dox (Fig. 7A). Double transgenic flies (rtTA(2)C1 × 7TAdh(2)A2) expressed high levels of β-gal in response to 0.1 mg/ml dox (Fig. 4), and this expression also had no detectable negative effects on lifespan (Fig. 7A). Finally, a different combination of transactivator and reporter were tested. Transactivator rtTA(X)A1 was crossed to reporter 7TAdh(3)D1 and age-synchronized cohorts of adult flies were treated throughout their adult lifespan with no dox, 0.01 mg/ml dox, or 1.0 mg/ml dox (Fig. 7B). Treatment with 1.0 mg/ml dox was found to have a negative affect on life span. However, treatment with 0.01 mg/ml dox had no detectable affect on life span. The same results were obtained with several other combinations of rtTA and reporter lines (data not shown). Since 0.01 mg/ml dox and 0.1 mg/ml dox allow high-level induction of β-gal (Fig. 4), and have no detectable effect on lifespan (Fig. 7), these results suggest that the system should be useful for assaying the effects on life span of overexpression of specific genes.

Discussion

The hybrid transcriptional activator (rtTA) consisting of the rtR fused to the transcriptional activation domain of the herpes virus protein VP16 has previously been shown to be capable of supporting dox-induced transcription in transgenic mice. The experiments presented

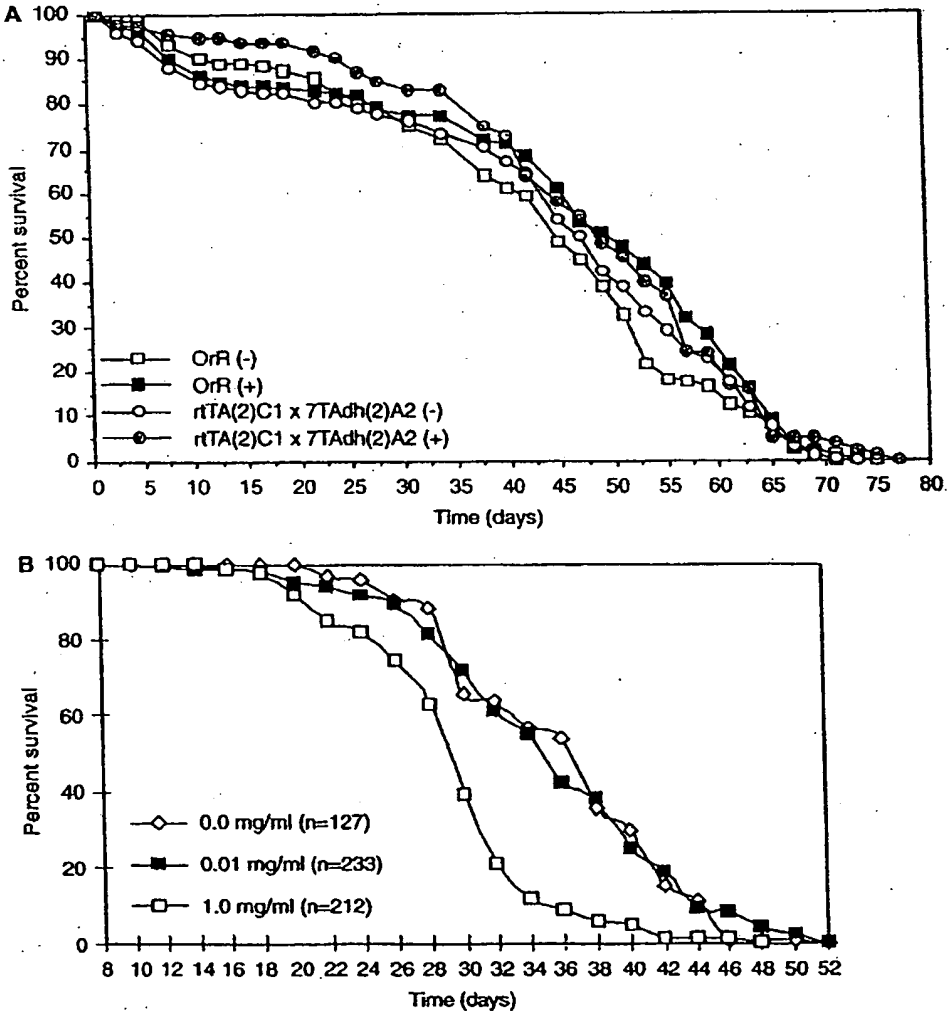

Fig. 7A, B Affect of dox-induced β-gal expression on *Drosophila* adult life span. A Wild-type Oregon R strain flies were treated throughout their adult lifespan either without dox (*open squares*) or with 0.1 mg/ml dox (*filled squares*), at 25° C. Flies were fed or mock-fed dox for 2 days, and then allowed to recover on standard media for 2 days, and this regimen was repeated until all the flies had died. The percentage of flies surviving is plotted as a function of time in days. The same experiment was performed using progeny from cross rtTA(2)C1 × 7TAdh(2)A2 grown in the absence of dox (*open circles*) or in the presence of 0.1 mg/ml dox (*closed circles*). At least 200 flies were used for each of the four survival curves. B Progeny from the cross rtTA(X)A1 × 7 TAdh(3)D1 were grown throughout their adult life span in the absence of dox (*open diamonds*), or in the presence of 0.01 mg/ml dox (*filled squares*), or 1.0 mg/ml dox (*open squares*), at 25° C. The number of flies used (n) for each survival curve is indicated. The different genotypes assayed in A and B vary in life span relative to each other, which is not unexpected due to the large effects of genetic background on life span (Curtsinger et al. 1995; Tower 1996)

here demonstrate that this rtTA functions in transgenic *Drosophila*, and can activate transcription at both the *hsp70* and *Adh* core promoters when they are linked to tetO sequences. Since all three reporter constructs performed similarly, the results suggest that in this system there is no significant difference in the effectiveness of the SV40 poly(A) signal relative to the *hsp70* poly(A) signal, and no significant difference between the effectivness of the *Adh*, *hsp70* and *Ubx* 5′ UTR regions. Dox-induced transgene expression was detected in all tissues, and induction ranged from 10- to 100-fold. Different transgenic lines containing the rtTA construct varied considerably in activity. Variation was observed in the maximal level of induction achieved, and in the amount of background activity observed in the absence of dox. This variation is likely to be due to chromosomal position effects on the expression of the rtTA transposon, and perhaps to other differences in the genetic background of the lines. The different transgenic lines of the reporter constructs also varied in activity, most probably for the same reasons. The maximum degree of induction that was achieved was 100-fold. This is dramatically less than the five orders of magnitude induction obtained with the tet-on system in transgenic mice (Kistner et al. 1996). The maximal induction achieved in *Drosophila* is limited by at least two factors: First, the reporter constructs are slightly leaky, in that variable, low-level β-gal activity is detected even in the absence of the rtTA transactivator. Second, the rtTA transactivator appears to be partially active even the absence of dox treatment, in that reporter plus transactivator was often more active than reporter alone. The first problem might be addressed by protecting the reporter constructs from position effects with insulator elements (Roseman et al. 1993), and/or by identifying a less leaky core promoter. The second problem can be mitigated by identifying particular rtTA lines, such as rtTA(3)E2, which exhibit less background activation. Finally, we hypothesize that the herpes virus VP16 transcriptional activation domain used to create the rtTA transactivator may be better suited to interaction with the mammalian transcriptional machinery than with the *Drosophila* transcriptional machinery. This possibility may also be relevant to the slower time course of induction observed in *Drosophila*.

Despite its limitations, this inducible system has several potential advantages relative to the use of heat shock gene promoters. The dox-inducible system should be useful for studying hsps, as it will allow the investigator to induce the expression of a single hsp, and potentially inhibit its expression with antisense RNA, without inducing the endogenous heat shock response. The dox- inducible system also allows the investigator to induce a gene of interest at any time during the life cycle. This is particularly relevant to study of the aging process (Curtsinger et al. 1995; Tower 1996), where it is often desirable to alter gene expression specifically in the adult. For example, constitutive over-expression of Cu/Zn SOD may have beneficial effects on *Drosophila* life span but it also appears to have toxic affects during pupal development (Reveillaud et al. 1991). Using the dox-inducible system it should be possible to avoid toxic effects during development and cause over-expression of transgenes specifically in the adult where beneficial effects on life span may be more apparent.

The dox-inducible system should be readily adaptable to tissue-specific induction. Replacement of the constitutive *Actin5C* promoter in the transactivator construct rtTA with a tissue-specific promoter should provide tissue-specific expression of the rtTA transactivator and thus tissue-specific induction of the reporter. An elegant system for tissue-specific expression of the yeast GAL4 transactivator has been developed for *Drosophila* (Brand and Perrimon 1993; Brand and Dormand 1995). In this case tissue-specific expression of the GAL4 transactivator is driven by an "enhancer-trap" system: the transactivator is under the control of a weak transcriptional promoter which can become activated in a tissue- and temporal-specific manner when the P element inserts near transcriptional enhancer sequences in the chromosome. The large variety of tissue- and temporal-specific GAL4 transactivator expression patterns generated thus allows tissue- and temporal-specific expression of "reporter" type constructs containing GAL4 binding sites in their promoters. This system could be adapted to drive expression of the rtTA transactivator, thus creating a large variety of tissue-specific expression patterns inducible by dox.

Finally, it may be possible to create dox-dependent mutations in *Drosophila*. P element constructs with transcriptional promoters directed out of the end of the P element can cause over-expression and/or misexpression of genes near the site of insertion, sometimes causing dominant mutations (Rorth 1996; Hay et al. 1997). Creation of a P element with a dox-inducible promoter directed out of the P element into flanking DNA sequences should sometimes cause dox-induced over-expression of a gene near the insertion site. This method should thus yield conditional (dox-dependent), dominant, gain-of-function mutations which would be useful for many types of genetic analyses; such experiments are now underway.

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